DEVELOPMENTAL BIOLOGY **203,** 424–434 (1998)
ARTICLE NO. DB988947

Centrosome Reduction during Mouse Spermiogenesis

G. Manandhar,* P. Sutovsky,* H. C. Joshi,† T. Stearns,‡ and G. Schatten*,[1]

**Departments of Obstetrics & Gynecology and Cell & Developmental Biology, Oregon Regional Primate Research Center, Oregon Health Science University, Beaverton, Oregon 97006; †Department of Cell Biology, Emory University, Atlanta, Georgia 30322; and ‡Department of Biological Sciences, Stanford University, Stanford, California 94304-5020*

The sperm does not contribute the centrosome during murine fertilization. To determine the manner in which a functional centrosome is reduced, we have studied centrosome degeneration during spermiogenesis of mice. The round spermatids display normal centrosomes consisting of a pair of centrioles along with γ-tubulin containing foci. However, they do not seem to organize microtubules. Elongating spermatids display γ-tubulin spots in the neck region, while microtubules are organized from the perinuclear ring as the manchette. Electron microscopic studies using immunogold labeling revealed that γ-tubulin is mainly localized in the centriolar adjunct from which an aster of microtubules emanates. Microtubules repolymerized randomly in the cytoplasm after nocodazole treatment and reversal. γ-Tubulin dissociates from the neck region and is discarded in the residual bodies during spermiation. The distal centriole degenerates during testicular stage of spermiogenesis, while the proximal centriole is lost during epididymal stage. Loss of centrosomal protein and centrioles in mouse sperm further confirm the maternal inheritance of centrosome during murine fertilization.

Key Words: **centrosome, centrioles, centriolar adjunct, γ-tubulin, spermiogenesis.**

INTRODUCTION

A typical centrosome of an animal cell consists of a pair of orthogonally oriented centrioles (Robbins *et al.*, 1968) surrounded by a cloud of electron dense fibrillar material (Gould and Borisy, 1977). Several types of proteins are found in the centrosome and some of them are involved in microtubule nucleation (Kuriyama, 1992; Kimble and Kuriyama, 1992). Centrosomes play a crucial role in organizing cytoplasmic microtubules during interphase and bipolar spindles during division. Therefore, the number of centrosome in cells is strictly regulated in each cell cycle through the processes of duplication during interphase and separation of the duplicated centrosomes in mitosis.

The normal cycle of centrosome propagation is interrupted during sexual reproduction. In the process of fertilization the cellular contents of the male and female gametes are pooled up and redistributed (Schatten, 1994). The centrosome must be reduced during gametogenesis to avoid doubling in the successive generation. Whereas the genome is equivalently reduced in male and female gametes, centrosome reduction seems to follow different modes in them. In most of the animals, mature sperm retain centrioles (Fawcett and Phillips, 1969; LeGuen and Crozet, 1989; Crozet, 1990; Sathananthan *et al.*, 1996; Sutovsky *et al.*, 1996a,b) which are duplicated before each cleavage (Paweletz *et al.*, 1987; Sathananthan *et al.*, 1996; Sutovsky *et al.*, 1996a) and propagated to the daughter cells. Centrioles are typically inherited through paternal lineage. Although the centrioles are present in the sperm, they lack many microtubule nucleating proteins (Schatten *et al.*, 1986; Stearns and Kirschner, 1994; Felix *et al.*, 1994). Reciprocally, vertebrate oocytes lose centrioles (Szollosi, 1972; Szollosi and Ozil, 1991) but retain MTOC proteins. Thus, there is a remarkable disparity in centrosomal reduction during the formation of male and female gametes.

This mode of centrosomal reduction holds true in most of the animals, but surprisingly not in the rodents. Both the proximal and distal centrioles are lost in rat sperm

[1] To whom correspondence should be addressed. Fax: (503) 6143725. E-mail: schatten@ohsu.edu.

0012-1606/98 $25.00

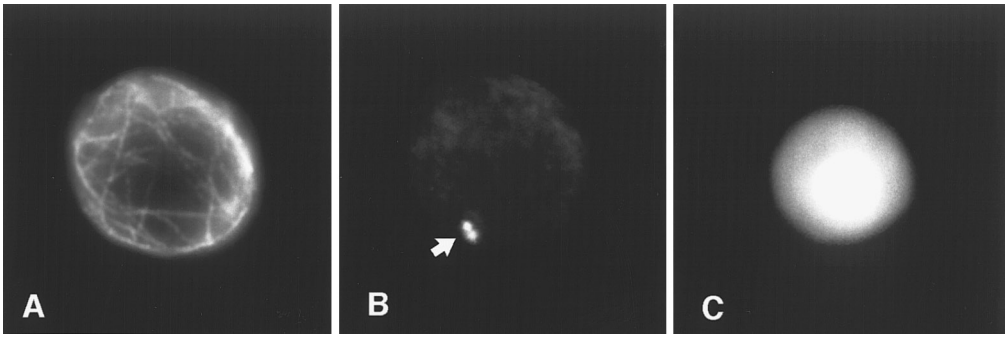

FIG. 1. Microtubule and γ-tubulin distributions in a round spermatid. (A) anti-β-tubulin labeling, (B) anti-γ-tubulin labeling; (C) DNA labeling with Hoechst 33258. The round spermatid displays unfocussed random cortical microtubules (A) and two γ-tubulin spots in the juxtanuclear region (broad white arrow in B).

(Woolley and Fawcett, 1973). Mouse sperm do not label with anticentrosomal autoimmune antibody 5051 (Schatten *et al.*, 1986) and do not form a sperm aster during the fertilization process (Schatten *et al.*, 1985). Whether centrioles are degenerated in them, as in rat sperm, is still unknown.

Centrosome reduction is still an unexplored phenomenon despite its vital role in maintaining normality of centrosomes in the progenies. An extreme case of centrosome reduction is seen in rodents—centriole degeneration has been shown in rat, while the loss of MTOC proteins has been shown in mice. The loss of centrioles and MTOC proteins may be interrelated processes in rodent spermiogenesis, nevertheless this relationship has never been shown. Moreover, the cellular pathway of the centrosomal reduction during the development of sperm is still largely unknown. In the present work we have pursued a comprehensive study of centrosome degenera-

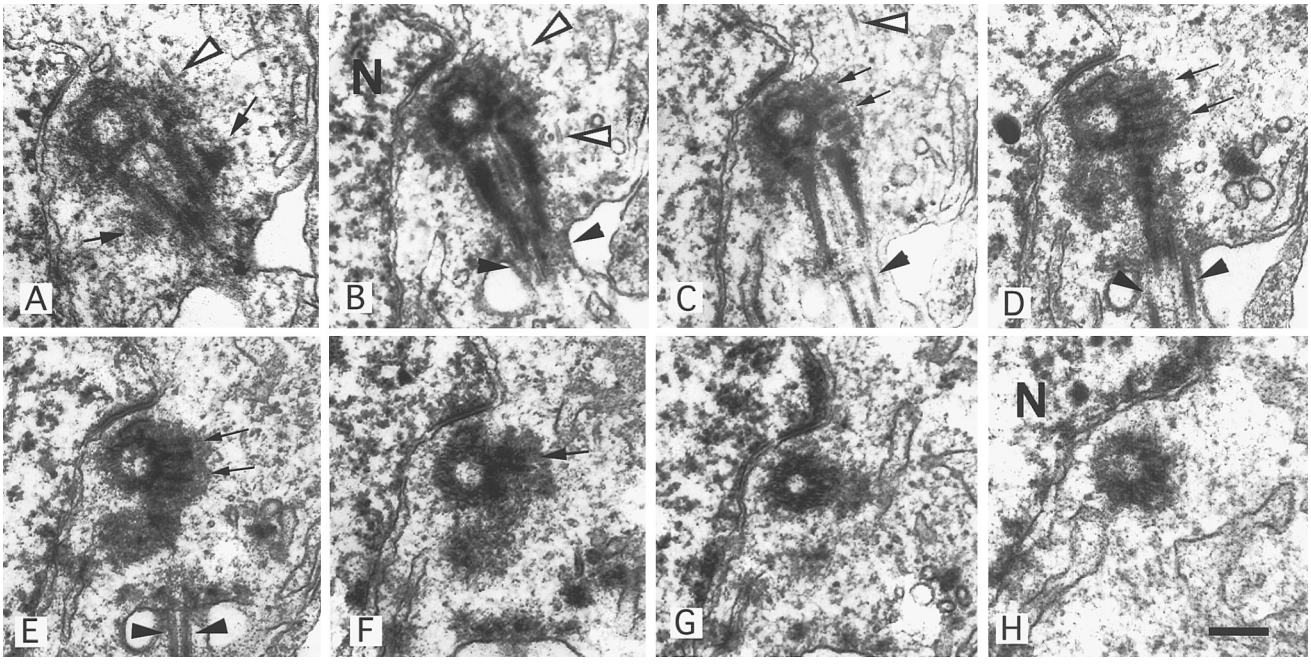

FIG. 2. Consecutive serial sections of the centriolar complex of a round spermatid. The distal centriole extends into an axoneme (black arrowheads in B–E) and is associated with an inconspicuous basal feet (arrows in A). The proximal centriole is associated with striated columns (arrows in C–F). Only one microtubule is seen at the vicinity of the centrioles (white arrowheads in A–C). N, nucleus. Bar, 0.25 μm.

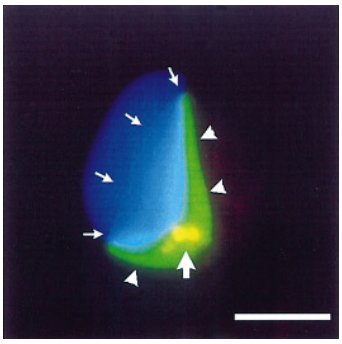

FIG. 3. Microtubule and γ-tubulin distributions in a midstage elongating spermatid. Red (appears yellow due to overlapping with the green signal from microtubules); γ-tubulin; green, microtubules; blue, DNA. The cell has well developed manchette (arrowheads), which is nucleated from an obliquely placed perinuclear ring (small arrows). The cell shows three γ-tubulin spots in the neck region (broad arrow), two probably corresponding to the centrioles and one to the proximal centriolar adjunct (see Fig. 4C). The manchette microtubule-nucleating region (small arrows) is not labeled with γ-tubulin. Bar, 5 μm.

tion during mouse spermiogenesis using electron microscopy and antibody against an important MTOC protein, γ-tubulin.

The haploid cells (1n) produced in seminiferous tubules after meiotic division of spermatocytes (2n) are the round spermatids. The overall developmental events forming mature sperm from spermatids is called the spermiogenesis. Round spermatids undergo a series of complicated morphological changes (Dooher and Bennett, 1973; Phillips, 1974), resulting in the formation of elongated and motile sperm. At the time of spermiation (i.e., when the developing sperm are released into the seminiferous tubule lumen) the developing sperm lose most of their cytoplasm in the form of residual body. In the murine testis, round spermatids require about 20 days to reach the spermiation stage, after the completion of meiosis (Oakberg, 1956; Clermont *et al.*, 1959).

MATERIALS AND METHODS

Isolation of Spermatids

Spermatids were isolated following the method described by Ogura and Yanagimachi (1993) and Goto *et al.* (1996). The seminiferous tubules were released in erythrocyte lysing buffer (ELB: NH_4Cl 155 mM, KH_2CO_3 10 mM, EDTA 3.2 mM, pH 7.2) by making a small incision at the caudal end of the testis and applying a gentle scooping pressure. The tubules were spread in the buffer to remove the red blood cells and most of the interstitial cells. They were washed with Dulbecco's modified Eagle medium (DME, Sigma) supplemented with 10 mM HEPES, 0.5% bovine serum albumin (fraction V, Sigma). The seminiferous tubules of two testes were minced in 1 ml of medium for 10 min with fine scissors. The suspension was diluted by adding another 1 ml of medium, pipetted for several times and then filtered through 20 μm mesh. The cells were pelleted by centrifuging at 500*g* for 5 min. The pellets were washed by resuspending in medium and repelleting, for two times. The isolation process was carried out at room temperature.

Immunofluorescence Microscopy

For simultaneous visualization of centrosome, microtubules, and DNA, the cells were labeled with anti-γ-tubulin, anti-β-tubulin antibodies, and Hoechst. The anti γ-tubulin antibody was produced in rabbit against the C-terminus of γ-tubulin, which is known to be conserved among phylogenetically diverse organisms (Joshi *et al.*, 1992; Stearns *et al.*, 1991; Stearns and Kirschner, 1994). The anti-β-tubulin was mouse monoclonal antibody (E7) obtained from the Developmental Studies, Hybridoma Bank (Iowa City, IA). The anti-γ-tubulin was diluted to 1:200 and the E7 antibody diluted to 1:10 with PBS and 1% NGS. The cells were attached to poly-L-lysine-coated coverslips, fixed with 2% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) for 1 h, and postfixed with cold methanol for 10 min. They were blocked with 10% normal goat serum (NGS) for 1 h and then incubated with a mixture of first antibodies for 1 h at 37°C. TRITC-conjugated anti-rabbit antibody and FITC-conjugated anti-mouse antibody (Zymed) were used as second antibodies, both at the dilution of 1:40 in PBS. DNA was stained with Hoechst 33258 dye. Immunofluorescent studies were conducted using Zeiss Axiophot microscope; images were digitally recorded using Metamorph software (Universal Imaging Corp., West Chester, PA), processed with Adobe Photoshop software (Adobe System Inc., Mountain View, CA), and printed in a Sony 8800 dye sublimation printer.

Nocodazole Treatment and Reversal

For nocodazole experiments, the seminiferous tubules were released directly in 1 ml of DME medium supplemented with Hepes and BSA without washing in ELB. The germ cells were isolated as before and cultured in Falcon petri dishes in DME supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and nocodazole (1 μg/ml, diluted from a 1 mM stock in DMSO, Sigma). Concentration of cells was 1×10^6/ml. Incubation was carried out in CO_2 incubator at 37°C. After 14 h, nocodazole was washed away and cells were allowed to recover in nocodazole free medium for 10 min.

Electron Microscopy

Seminiferous tubules and pieces of the epididymis were fixed with 2.5% glutaraldehyde in 0.1 M PBS for 2 h at room temperature. After thoroughly washing with PBS, the tissues were postfixed with 1% OsO_4 in PBS at 4°C for 3 h. They were washed with water for several times, dehydrated in an ascending ethanol series, profused with propylene oxide, and embedded in PolyBed 812. Thin sections were cut in Sorvall MT 2B ultramicrotome. They were collected on Pioloform-coated slot grids or 100 mesh grids, stained with uranyl acetate and lead citrate, and examined under a Phillips CM 120 transmission electron microscope.

Immunogold Electron Microscopy

The seminiferous tubules were fixed either with a mixture of 2% paraformaldehyde and 0.2% glutaraldehyde for 1 h, or in cold methanol (−20°C) for 20 min. The paraformaldehyde–glutaraldehyde-fixed

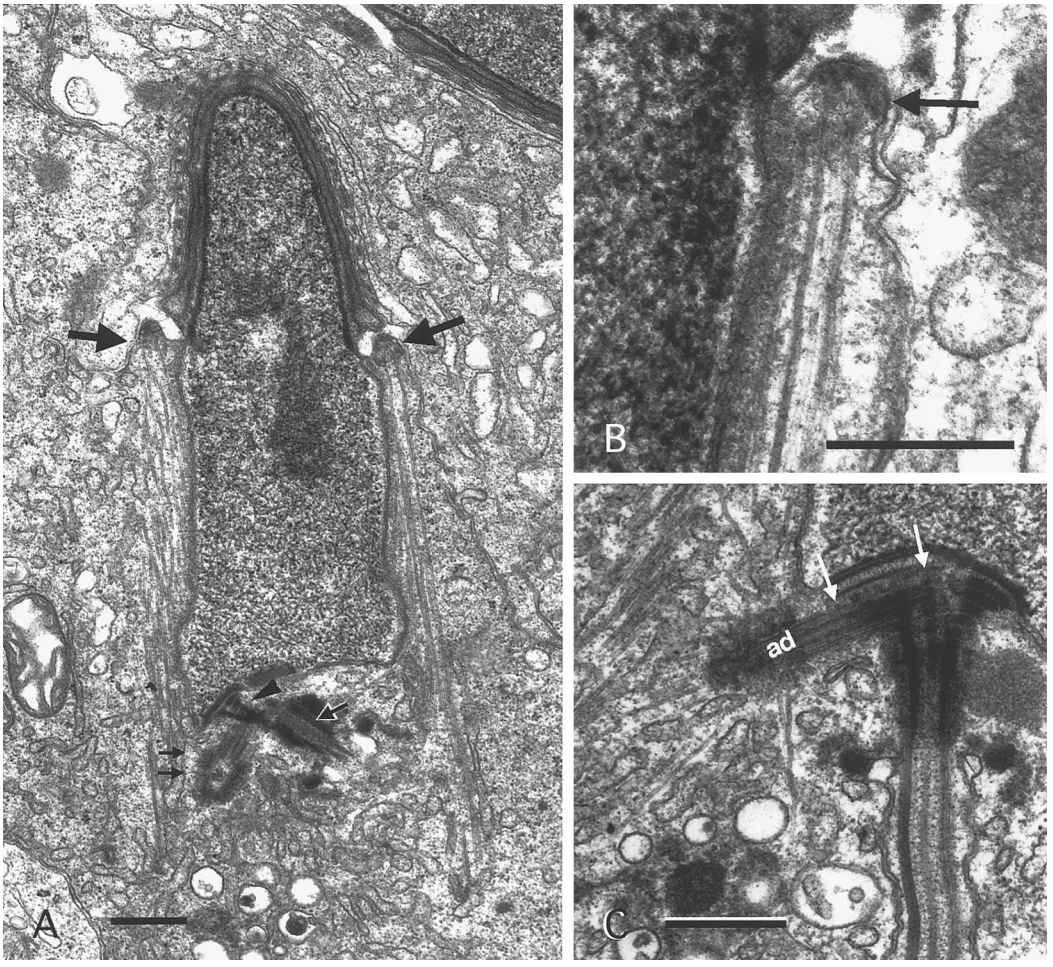

FIG. 4. The manchette and the centriolar complex of elongating spermatids. (A) A spermatid showing manchette microtubular bundles emanating from the perinuclear ring which is formed below the posterior edge of the acrosome (large arrows). The section has passed longitudinally through the distal centriole (small arrow) and the proximal centriolar adjunct (double arrows) in the neck region. The distal centriole is connected to the implantation fossa by striated columns (arrowhead). (B) Higher magnification of the manchette microtubule-nucleating region. This area comprises furrows of membranous folds in which electron-dense materials are accumulated. The distal end of microtubules is embedded in the electron-dense material (arrow). (C) The centriolar complex of an elongating spermatid. The microtubule structure of the centrioles is not discernible at this plane of section due to dense material accumulated around them. The proximal centriole (marked with arrows) has elongated as an adjunct (ad) which is capped with electron-dense fibrous materials. The adjunct nucleates an aster of microtubules. Bar, 0.5 μm.

tissues were washed and treated with 1 mg/ml $NaBH_4$ for 30 min, rapidly dehydrated in ethanol, shaken with LR-White:ethanol (1:1) for 1 h, and embedded in LR-White (London Resin Co., Ltd, England). The methanol fixed tissues were directly profused with the mixture of LR-White and ethanol. Polymerization was carried out at 60°C for 24 h. Appropriate stages of spermatids were selected in semithin sections. Silver-colored thin sections were collected on Pioloform-coated nickel grids which were incubated sequentially with 10% NGS (1 h), anti-γ-tubulin (1 h), and colloidal gold-conjugated anti-rabbit IgG (10 nm gold, Jackson ImmunoResearch Inc.), followed by postfixation with 2.5% glutaraldehyde (20 min) and staining with uranyl acetate (10 min).

The negative photographs were scanned with a Kodak Leafscan-35 image scanner, recorded on magneto-optical disks, processed with Adobe Photoshop 4.0 software, and printed by a Sony 8800 dye sublimation printer.

RESULTS

Centrosomes of Mouse Spermatids Have Centrioles and γ-Tubulin but Do Not Nucleate Microtubules

The early-stage spermatids have round shape and were easily distinguished from other round-shaped cells, due to their characteristic size and nuclear morphology (Leb-

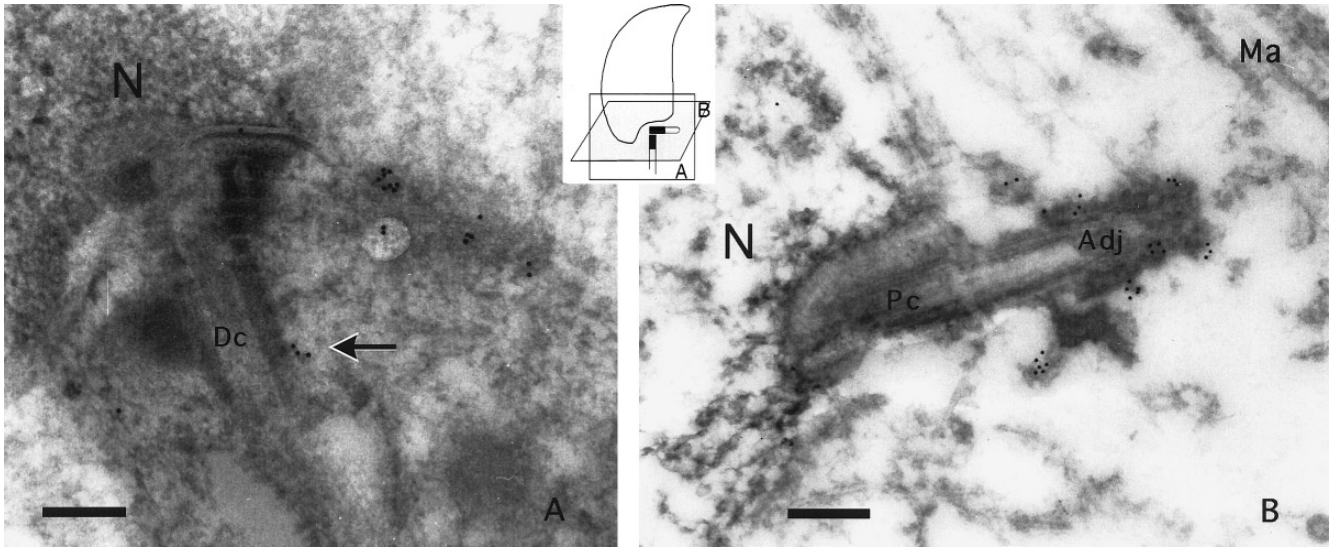

FIG. 5. Immunogold labeling of elongating spermatids with anti-γ-tubulin antibody. (A) A grazing section passing through the fibrillar material of the adjunct of a spermatid fixed with paraformaldehyde–glutaraldehyde. (B) A horizontal section passing through the proximal centriole and the adjunct of a spermatid fixed with methanol. The immunogold particles are bound mainly around the adjunct. The proximal region of the distal centriole also shows some gold particles (arrow in A). Inset, a schematic drawing of a sperm head showing the plane of sections of A and B. N, nucleus; Dc, distal centriole; Pc, proximal centriole; Adj, adjunct; Ma, manchette; Bar, 0.5 μm.

lond and Clermont, 1952). The microtubules of those cells were distributed as a random network in the cortical region without any clear focus (Fig. 1A). Anti-γ-tubulin labeling exhibited one or two spots in the juxtanuclear region (Fig. 1B). The cortical microtubule network did not seem to focus towards the γ-tubulin-labeled structures.

Electron microscopic studies of the round spermatids revealed two orthogonally oriented centrioles, associated with a thickened evagination of the nuclear envelope (Figs 2A–2H). The distal centriole possessed inconspicuous basal feet and functions as a basal body of an axoneme (Fig. 2A). Very few microtubules were observed at the vicinity of the centriolar complex (Figs 2A–2C).

During the stage when the nucleus begins elongation, the random microtubular network is replaced by a manchette. γ-Tubulin immunofluorescence reveals two spots in the neck region. Some spermatids display an additional spot seemingly associated with one of the spots (Fig. 3). Some late-stage elongating spermatids with recently disorganized manchette display an aster of microtubules associated with one of the γ-tubulin spots (see Figs. 8A, 8B). The manchette microtubules are nucleated from perinuclear area (Fig. 3; large arrows in Figs. 4A and 4B), located well above the site of the γ-tubulin spots. The area of manchette microtubules does not display γ-tubulin labeling (Fig. 3).

Electron microscopy of the elongating spermatids shows a deep membranous groove around the equatorial region of the nucleus, from where the manchette is nucleated (Fig. 4A). In this area, the microtubular ends are deeply embedded in the fibrous material (Fig. 4B). A remarkable feature of the centriolar complex of the elongating spermatids is the development of an adjunct from the proximal centriole. It is capped with electron-dense fibrous structure, which nucleates microtubules (Fig. 4C). The γ-tubulin spots showing microtubule nucleation in some spermatids (see Figs 8A and 8B), are likely to be the adjuncts. Microtubule nucleation from the proximal or the distal centrioles was not observed in ultrastructural studies.

Immunogold electron microscopy of the elongating spermatids revealed that γ-tubulin is associated with the centrioles and the adjunct (Fig. 5), corresponding to the punctate spots observed in immunofluorescence studies (Fig. 3). Remarkably higher immunoreactivity was observed in the fibrillar material of the adjunct, the structure which emanates microtubules (Fig. 4C).

Microtubules, Repolymerized after Nocodazole Treatment, Do Not Nucleate from γ-Tubulin

Microtubules depolymerized completely in the round spermatids treated with nocodazole for 14 h. After 10 min recovery, microtubules reappeared as a random network which did not show any association with the centrosome, as identified with γ-tubulin labeling (Fig. 6A). This observation further strengthens the hypothesis that centrosomes of round spermatids do not participate in mic-

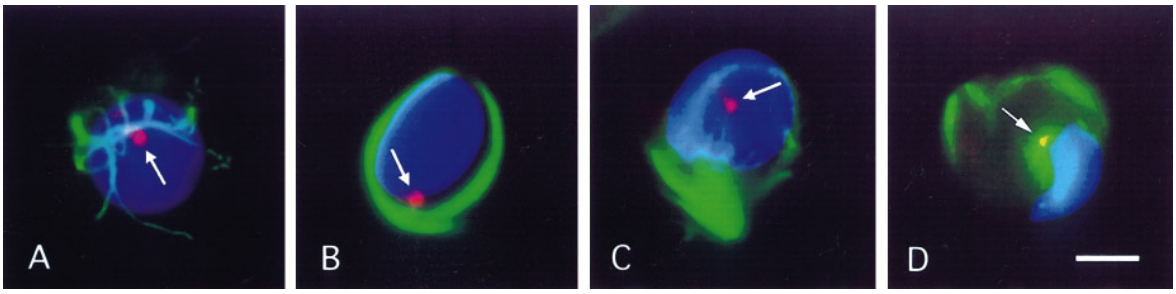

FIG. 6. Microtubule recovery in spermatids after nocodazole treatment. Red, γ-tubulin; green, microtubules; blue, DNA. (A) A round spermatid showing randomly repolymerized microtubules in the cortical region. The γ-tubulin (arrow) is not involved in microtubule nucleation in the cell. (B) An early-stage elongating spermatid in which microtubules have been polymerized in the perinuclear area without the participation of γ-tubulin (arrow). (C) An early-stage elongating spermatid showing repolymerization of microtubule bundles far from the γ-tubulin spot (arrow). (D) An elongating spermatid in which repolymerized microtubules are randomly distributed in the cytoplasm. The spermatid possesses an aster which has been nucleated by the γ-tubulin spot of the neck region (arrow). The developmental stage of the spermatid is similar to the one shown in Fig. 3. Bar, 5 μm.

rotubule nucleation. In the elongating spermatids microtubules repolymerized as bundles, asters, or network, randomly distributed in the cytoplasm (Figs 6A–6D). In some early-stage elongating spermatids, microtubule polymerization was observed in the perinuclear area (Fig. 6B). In a few elongating spermatids, small asters were found to be nucleated by the γ-tubulin spots which probably represent the centriolar adjuncts (Fig. 6D).

γ-Tubulin Is Lost from the Sperm during Spermiation

γ-Tubulin spots were observed at the base of the tail until the late stage of spermatid development. At the time of spermiation, γ-tubulin apparently dissociates from that region and shed along with the residual bodies. The residual bodies contained punctate (arrows: Fig. 7A), as well as

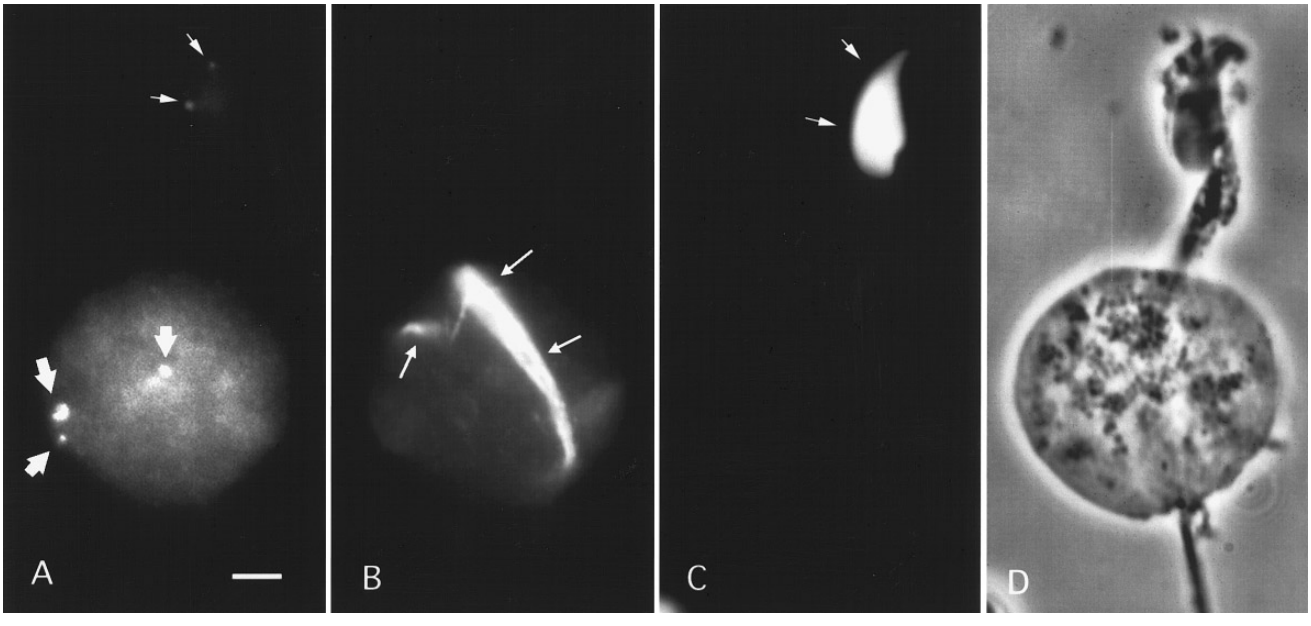

FIG. 7. γ-Tubulin and microtubule loss from spermatid at the spermiation stage. (A) Anti-γ-tubulin labeling, (B) Anti-β-tubulin labeling, (C) Hoechst 33258 labeling of DNA, (D) phase-contrast image. The γ-tubulin has dissociated from the neck region and randomly distributed in the residual body as punctate spots (broad white arrows in A) or diffuse labeling. The head region of this spermatid shows two small punctate γ-tubulin spots (arrows in A and C). The residual body also has few bundles of microtubules (arrows in B). Bar, 5 μm.

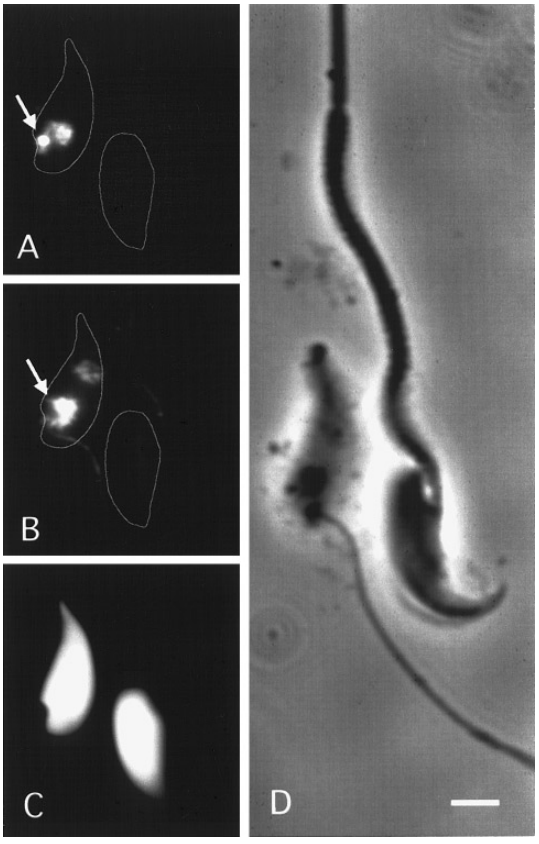

FIG. 8. Mouse testicular sperm showing loss of γ-tubulin and microtubules in advance stage. (A) Anti-γ-tubulin labeling, (B) anti-β-tubulin labeling, (C) Hoechst 33258 labeling of DNA, (D) phase-contrast image. A–C show the head region of two sperm (marked with white lines). The developmental stage of the right sperm is more advanced than that of the left one as it is apparent from a conspicuously thicker mid-piece. The neck region of the left sperm has two γ-tubulin spots, one of which emanates a small aster of microtubules (arrows in A and B). An advanced stage sperm (right) lacks γ-tubulin or microtubule. Bar, 5 μm.

diffuse γ-tubulin labeling. A few bundles of microtubules were also found in the residual bodies but not associated with the γ-tubulin spots (Figs. 7A and 7B). In some testicular sperm, γ-tubulin spots were found in the head region (Fig. 7A). γ-Tubulin was not detected in the late stage testicular sperm (Fig. 8) or in the epididymal sperm.

Centrioles Degenerate in the Mature Sperm

The distal centrioles disintegrate during testicular stage, and by the time the sperm reach epididymis, they are completely lost (Fig. 9). The space previously occupied by the distal centriole is surrounded by the columns of connecting pieces. Whereas the distal centrioles are lost during testicular stage, the proximal centrioles degenerate in the epididymis. Various stages of degeneration of the proximal centriole were observed in the adjacent sperm or in the sperm showing similar stages of maturation (Figs. 9A–9C). These observations suggest that the stages of the proximal centriole degeneration are not synchronous among the adjacent sperm and do not correspond to their maturation stages. In some epididymal sperm, short microtubules stubs were observed deeply embedded in the centriolar vault (Fig. 9B). Finally, in fully mature epididymal sperm, the proximal centriole is also completely degenerated (Fig. 9C). The vault around it usually collapses (Fig. 9C).

DISCUSSION

Centrosome is reduced in mammalian sperm in general, but mouse sperm show complete elimination of centrosomal components. The present study reveals three stages of centrosome reduction during mouse spermiogenesis: (i) loss of microtubule nucleating function, (ii) dissipation of residual γ-tubulin, and (iii) degeneration of centrioles (Fig. 10).

Loss of Microtubule-Nucleating Function—An Early Stage of Centrosome Reduction

Although the centrosomes of mouse spermatids are apparently normal, consisting of a pair of centrioles and γ-tubulin, they do not nucleate detectable microtubular array. These observations signify the loss of microtubule nucleating function by the centrosomes which could be an early manifestation of centrosome reduction taking place during spermiogenesis. It is accomplished before the centrioles are degenerated or γ-tubulin is completely dissociated from them. Analogous phenomenon was observed during myogenic differentiation (Tassin *et al.*, 1985). The loss of microtubule nucleating function of the centrosome may not prevail in terminal differentiation in general, particularly in those cases, in which the centrosome is not degenerated. For example, centrosomes nucleate microtubules in neural cells (Bartlett and Banker, 1984; Stevens *et al.*, 1988), mouse cochlear epithelial cells (Mogensen *et al.*, 1997), and other ciliated epithelial cells (review in Joshi, 1993).

Inactive γ-Tubulin of Centrosomes

γ-Tubulin is involved in microtubule nucleation in a variety of cellular systems (review in Joshi, 1993). Recent studies have shown that γ-tubulin is conjugated with six types of proteins forming a ring-shaped complex called the γ-tubulin ring complex (γ-TuRC) which directly nucleates microtubules (Zheng *et al.*, 1995; Moritz *et al.*, 1995; Raff, 1996). The mouse spermatids entail an intriguing scenario: The γ-tubulin associated with the distal and the proximal centrioles do not nucleate microtubules, while those associated with the centriolar adjunct form an aster of microtubules (Figs. 3–5). We propose two alternative explanations to account for the existence of active and inactive forms of γ-tubulin in the centriolar apparatus of the spermatids. The

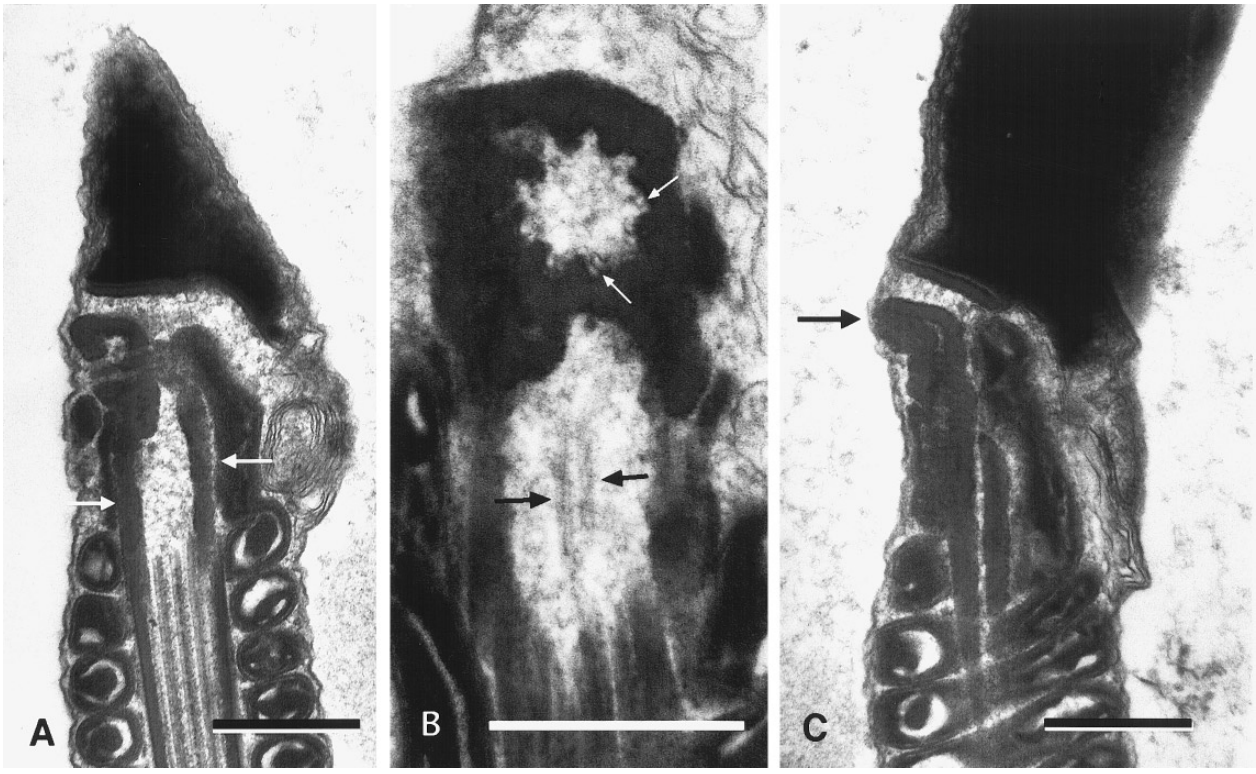

FIG. 9. Degeneration of centrioles in the epididymal sperm. (A) Section passing longitudinally through the distal and the proximal centriolar regions. The distal centriole has degenerated and the space is surrounded by the outer dense fibers (arrows). Few remnants of the centriolar microtubules are visible in the proximal centriolar vault while the majority of them has disappeared. The upper margin of the centriolar vault has folded back into the lumen. (B) Section passing transversely through the proximal centriolar vault and obliquely through the distal centriolar region. The extended central microtubule doublet of the axoneme (black arrows) is visible in the distal centriolar vault. The proximal centriole is in the final stage of degeneration. Few remnants of microtubules (white arrows) are visible in the finger-like projections, which are the remnants of the spaces previously occupied by the microtubular triplets. (C) Section passing longitudinally through the proximal centriolar vault of a mature epididymal sperm. The proximal centriole has been lost completely and its vault has collapsed (arrow). Bar, 0.5 μm.

first possibility invokes a necessity of a protein factor for microtubule nucleation by γ-tubulin. Several indirect evidences suggest that γ-tubulin alone cannot nucleate microtubules (review in Masuda and Shibata, 1996). For example, inactive γ-tubulin, present in the spindle pole body of interphase yeasts, does not nucleate microtubules (Horio *et al.,* 1991; Masuda *et al.,* 1992), possibly due to the lack of protein factor (Masuda and Shibata, 1996). Analogous situation in mouse spermatids, however, is less likely because we found inactive γ-tubulin spot in the close vicinity of microtubule aster, and there is no obvious reason why the protein factor should be unavailable to the γ-tubulin spot. An alternative possibility envisages a specific γ-TuRC-binding factor that could be selectively present in the adjunct. Inability of the centriolar proper to bind the microtubule-nucleating complex, γ-TuRC, could be due to the lack of the factor in their matrix. The role of such protein factors in microtubule nucleation and binding γ-TuRC to the centrosomal matrix is currently under investigation in some laboratories (Feldman and Stearns, 1996; Moritz *et al.,* 1996). Although the nature of the factor is not yet fully clear, preliminary data show that it is present in the centrosomes of *Xenopus* sperm and *Drosophila* embryo cells, and can be dissociated with chaotropic agents (Feldman and Stearns, 1996; Moritz *et al.,* 1996). The loss of the protein factor abolishes the ability of the sperm to form functional MTOC. Our study suggests that the centriolar complexes of mouse spermatids lose affinity to these protein factors in the process of degeneration. However, nascent γ-tubulins still bind to the centriolar complexes without the specific binding factor; yet they are incapable of nucleating microtubules. The loss of microtubule nucleating function by G0 centrioles was shown in some earlier cell fusion experiments (Peterson and Berns, 1979; Szollosi *et al.,* 1986; Manandhar and Onishchenko, 1995).

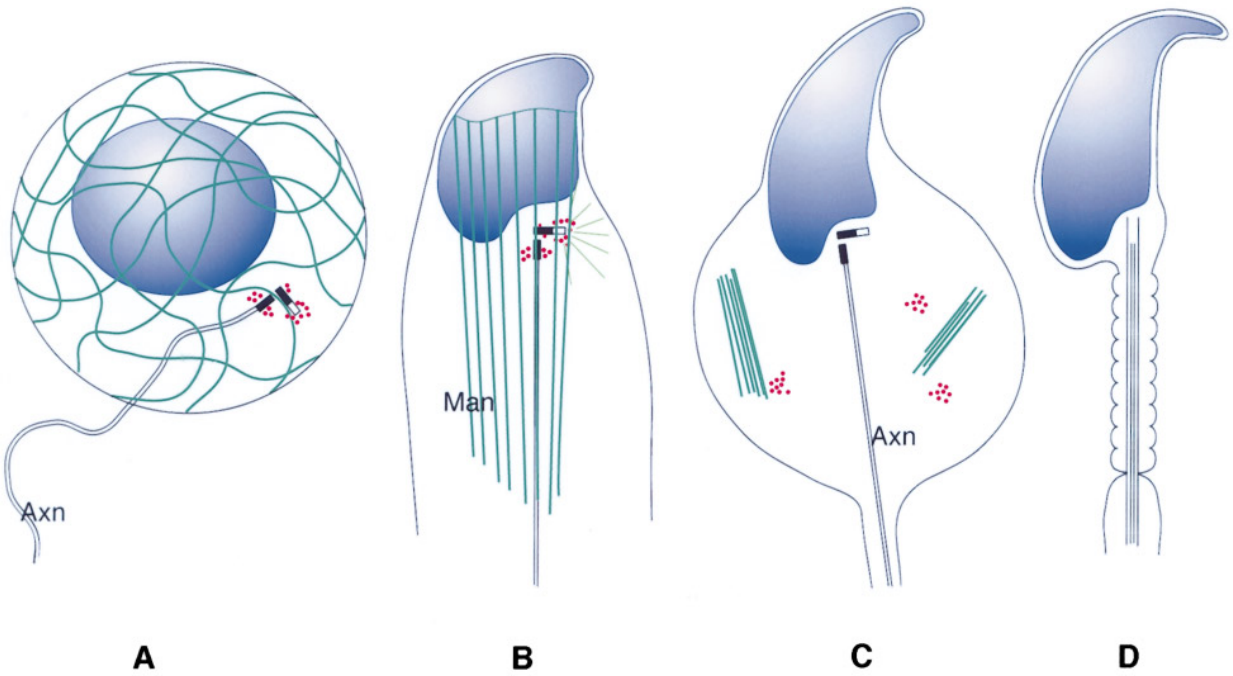

FIG. 10. Schematic diagram showing the stages of centrosome loss during mouse spermiogenesis. Centrosome of developing spermatids comprises two orthogonally oriented centrioles and γ-tubulin (A, B) but do not function as MTOCs of the microtubular systems of spermatids. The cortical microtubule network of the round spermatid (A) and the manchette of elongating spermatids (B) do not nucleate from the centrosome. However, the proximal centriolar adjunct of the elongating spermatids possesses γ-tubulin and nucleates microtubule aster (B). During spermiation, the γ-tubulin dissociates from the pericentriolar region and is discarded in the residual bodies (C). During the final stage of spermiogenesis, both the distal and the proximal centrioles disintegrate. The axoneme and the distal centriolar regions are surrounded by the dense fibers (D). Blue, nucleus; green, microtubules; red, γ-tubulin; solid black cylinders, centrioles; open cylinder, centriolar adjunct; Axn, axoneme; Man, manchette.

Loss of Residual γ-Tubulins—The Second Stage of Centrosome Reduction

Whereas the loss of microtubule-nucleating function of the centriolar complex comprises the first stage of centrosomal reduction, dispersion of the residual γ-tubulin from the centriolar matrix and its loss to the residual body is the second stage of this process. Seemingly, the dissociation of γ-tubulin from the neck region correlates with the centriole degeneration. However, this mechanism is unlikely, because γ-tubulin loss takes place during the spermiation stage, whereas centriole disintegration is completed much later, at the epididymal stage. Moreover, γ-tubulin has not been detected in *Xenopus* sperm (Stearns and Kirschner, 1994; Felix *et al.*, 1994), which possess centrioles (Bernardini *et al.*, 1986; Felix *et al.*, 1994).

Centriolar Disintegration—The Final Stage of Centrosome Reduction

The final event of the centrosome reduction is centriole degeneration itself. In mouse sperm, the distal centriole degenerates in the testes and the proximal one in the epididymis. It should be noted that the degeneration process is asynchronous and does not correlate with the age of the sperm. Therefore, the possibility cannot be excluded that some sperm with a remnant of proximal centriole and γ-tubulin exist in epididymis (Schatten *et al.*, 1985; Palacios *et al.*, 1993). Except for minor differences in the fine structure, the mouse sperm are similar to those of rat, regarding the loss of both centrioles.

ACKNOWLEDGMENTS

G.M. was partially supported by the MCBN/UNESCO Postdoctoral Fellowship (Project No. 266). We are very thankful to Joel H. Ito for art work and to Calvin Simerly for helpful comments and advice. The support of the ORPRC from RR 00163 and by research grants from the NICHD (HD 12913, HD 32887) to G. Schatten is gratefully acknowledged.

REFERENCES

Bartlett, P. W., and Banker, G. A. (1984). An electron microscopic study of the development of axons and dendrites by hypocampal neurons in culture. I. Cells which develop without intercellular contacts. *J. Neurosci.* **4,** 1944–1953.

Bernardini, G., Stipani, R., and Melone, G. (1986). The ultrastructure of *Xenopus* spermatozoon. *J. Ultrastruct. Mol. Struct. Res.* **94,** 188–194.

Clermont, Y., Leblond, C. P., and Messier, B. (1959): Durée du cycle de lépithelium seminal du rat. *Arch. Anat. Microsc. Morphol. Exp.* **48,** 37–56.

Crozet, N. (1990). Behavior of the sperm centriole during sheep oocyte fertilization. *Eur. J. Cell Biol.* **53,** 321–332.

Dooher, G. B., and Bennett, D. (1973). Fine structural observations on the development of the sperm head in the mouse. *Am. J. Anat.* **136,** 339–362.

Fawcett, D. W., and Phillips, D. M. (1969). The fine structure and development of the neck region of the mammalian spermatozoon. *Anat. Rec.* **165,** 153–184.

Feldman, D., and Stearns, T. (1996). Investigation into the mechanism of γ-tubulin recruitment to the sperm midpiece during fertilization. *Mol. Biol. Cell (Suppl.)* **7,** 1199a.

Felix, M., Antony, C., Write, M., and Maro, B. (1994). Centrosome assembly in vitro: Role of γ-tubulin recruitment in *Xenopus* sperm aster formation. *J. Cell Biol.* **124,** 19–31.

Goto, K., Kinoshita, A., Nakanishi, Y., and Ogawa, K. (1996). Blastocyst formation following intracytoplasmic injection of in vitro derived spermatids into bovine oocytes. *Hum. Reprod.* **11,** 824–829.

Gould, R. R., and Borisy, G. G. (1977). The pericentriolar material in Chinese hamster ovary cells nucleates microtubule formation. *J. Cell Biol.* **73,** 601–615.

Horio, T., Uzawa, S., Jung, M. K., Oakley, B. R., Tanaka, K., and Yanagida, M. (1991). The fission yeast γ-tubulin is essential for mitosis and is localized at microtubule organizing centres. *J. Cell Sci.* **99,** 693–700.

Joshi, H. C, Palacios, M. J., McNamara, L., and Cleveland, D. W. (1992). γ-Tubulin is a centrosomal protein required for cell cycle dependent microtubule nucleation. *Nature* **356,** 80–83.

Joshi, H. C. (1993). γ-tubulin: The hub of cellular microtubule assemblies. *BioEssays* **15,** 637–643.

Kimble, M., and Kuriyama, R. (1992). Functional components of microtubule organizing centers. *Int. Rev. Cytol.* **136,** 1–50.

Kuriyama, R. (1992). Monoclonal antibodies to microtubule-organizing center antigens. *In* ''Centrosome'' (V. I. Kalnins, Ed.), pp. 331–351. Academic Press, New York.

Leblond, C. P., and Clermont, Y. (1952). Definition of the stages of the cycle of the seminiferous epithelium in the rat. *Ann. NY Acad. Sci.* **55,** 548–573.

Le Guen, P., and Crozet N. (1989). Microtubule and centrosome distributions during sheep fertilization. *Eur. J. Cell Biol.* **48,** 239–249.

Manandhar, G., and Onishchenko, G. E. (1995). Centriolar cycle of fused cells. *J. Cell Sci.* **108,** 667–674.

Masuda, H., Sevik, M., and Cande, W. Z. (1992): In vitro microtubule-nucleating activity of spindle pole bodies in fission yeast *Saccharomyces pombe:* Cell cycle dependent activation in *Xenopus* cell-free extracts. *J. Cell Biol.* **117,** 1055–1066.

Masuda, H., and Shibata, T. (1996): Role of γ-tubulin in mitosis-specific microtubule nucleation from the *Schizosaccharomyces pombe* spindle pole body. *J. Cell Sci.* **109,** 165–177.

Mogensen, M. M., Mackie, J. B., Doxsey, S. J., Stearns, T., and Tucker, J. B. (1997). Centrosomal deployment of γ-tubulin and pericentrin—Evidence for a microtubule-nucleating domain and a minus-end docking domain in mouse epithelial cells. *Cell Motil. Cytoskelet.* **36,** 276–290.

Moritz, M., Braunfeld, M. B., Fung, J. C., Sedat, J. W., Alberts, B. M., and Agard, D. A. (1995). Three-dimensional structural characterization of centrosomes from early *Drosophila* embryos. *J. Cell Biol.* **130,** 1149–1159.

Moritz, M., Zheng, Y., and Alberts, B. M. (1996). Reconstitution of microtubule nucleation by the centrosome. *Mol. Biol. Cell* **7**(Suppl.), 1201a.

Oakberg, E. F. (1956). Duration of spermiogenesis in the mouse and timing of stages of the cycle of the seminiferous epithelium. *Am. J. Anat.* **99,** 507–516.

Ogura, A., and Yanagimachi, R. (1993). Round spermatid nuclei injected into hamster oocytes form pronuclei and participate in syngamy. *Biol. Reprod.* **48,** 219–225.

Palacios, M. J., Joshi, H. C., Simerly, C., and Schatten, G. (1993). γ-tubulin reorganization during mouse fertilization and early development. *J. Cell Sci.* **104,** 383–389.

Paweletz, N., Mazia, D., and Finze, E. (1987). Fine structural studies of the bipolarization of the mitotic apparatus in the fertilized sea urchin eggs. I. The structure and behavior of centrosomes before fusion of the pronuclei. *Eur. J. Cell Biol.* **44,** 195–204.

Peterson, S. P., and Berns, M. W. (1979). Mitosis in flat PTK_2-human hybrid cells. *Exp. Cell Res.* **120,** 223–136.

Phillips, D. M. (1974). ''Spermiogenesis.'' Academic Press, New York.

Raff, J. W. (1996). Centrosomes and microtubules: Welded with a ring. *Trends Cell Biol.* **6,** 248–251.

Robbins, E., Jentzsch, G., and Micali, A. (1968). The centriole cycle in synchronized HeLa cell. *J. Cell Biol.* **36,** 329–339.

Sathananthan, A. H., Ratnam, S. S., Ng, S. C., Tarim, J. J., Gianaroli, L., and Trounson, A. (1996). The sperm centriole: Its inheritance, replication and perpetuation in early human embryos. *Hum. Reprod.* **11,** 345–356.

Schatten, G. (1994). The centrosome and its mode of inheritance: The reduction of the centrosome during gametogenesis and its restoration during fertilization. *Dev. Biol.* **165,** 299–335.

Schatten, G., Simerly, C., and Schatten, H. (1985). Microtubule configurations during fertilization, mitosis and early development in the mouse and the requirement for egg microtubule-mediated motility during mammalian fertilization. *Proc. Natl. Acad. Sci. USA* **82,** 4152–4156.

Schatten, H., Schatten, G., Mazia, D., Belczon, R., and Simerly C. (1986). Behavior of the centrosome during fertilization and cell division in mouse oocytes and sea urchin eggs. *Proc. Natl. Acad. Sci. USA* **83,** 105–109.

Stearns, T., Evans, L., and Kirschner M. (1991). γ-tubulin is a highly conserved component of the centrosome. *Cell* **65,** 825–836.

Stearns, T., and Kirschner, M. (1994). In vitro reconstitution of centrosome assembly and function: The central role of γ-tubulin. *Cell* **76,** 623–637.

Stevens, J. K., Trogadis, J., and Jakobs, R .J. (1988). Development and control of axial neurite from: A serial electron microscopic analysis. *In* ''Intrinsic Determinants of Neural Form and Function'' (R. J. Lasek and M. M. Black, Eds), pp. 115–146. A. R. Liss, New York.

Sutovsky, P., Hewitson, L., Simerly, C., Tengowski, M. W., Navara, C. S., Haavisto, A., and Schatten, G. (1996a). Intracytoplasmic sperm injection for rhesus monkey fertilization results in unusual chromatin, cytoskeleton and membrane events but eventually leads to pronuclear development and sperm aster assembly. *Hum. Reprod.* **11,** 1703–1712.

Sutovsky, P., Navara, C. S., and Schatten, G. (1996b). Fate of the sperm mitochondria, and the incorporation, conversion and disassembly of the sperm tail structures during bovine fertilization. *Biol. Reprod*. **55,** 1195–1205.

Szöllosi, D. (1972). Changes of some cell organelles during oogenesis in mammals. *In* ''Oogenesis'' (J. D. Biggers, and A. Schuetz, Eds.), pp. 47–64. University Park Press, Baltimore.

Szöllosi, D., Czolowska, R., Soltynska, M. S., and Tarkowski, A. K. (1986). Ultrastructure of cell fusion and premature chromosome condensation of thymocyte nuclei in metaphase II mouse oocyte. *Biol. Cell*. **56,** 239–250.

Szöllosi, D., and Ozil, J. (1991). De novo formation of centrioles in parthenogenetically activated, diploidized rabbit embryos. *Biol. Cell* **72,** 61–66.

Tassin, A. M., Maro, B., and Bornens, M. (1985). Fate of microtubule-organizing centers during myogenesis in vitro. *J. Cell Biol.* **100,** 35–46.

Woolley, D. M., and Fawcett, D. W. (1973). The degeneration and disappearance of the centrioles during the development of the rat spermatozoon. *Anat. Rec.* **177,** 289–302.

Zheng, Y., Wong, M. L., Alberts, B., and Mitchison, T. (1995). Nucleation of microtubule assembly by a γ-tubulin-containing ring complex. *Nature* **378,** 578–583.

Received for publication February 11, 1998
Revised May 5, 1998
Accepted May 5, 1998